ISOLATION AND CHARACTERIZATION OF RAT LIVER NUCLEAR ENVELOPES

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Judy Christine Griffin

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by

Judy Christine Griffin

Approved by Committee:

Robert M. Kodama
Chairman

Harold D. Swanson

Robert W. Fuller

Earle L. Canfield
Dean of the School of Graduate Studies

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INTRODUCTION AND REVIEW OF THE LITERATURE

Introduction. The role of the nuclear envelope may be fundamental to the physiology of the cell (Kodama and Tedeschi, 1968). Evidence is found in the literature supporting an interrelated system of membranes within the cell, implying the continuity of the plasma membrane with the endoplasmic reticulum, the endoplasmic reticulum with the outer nuclear membrane, the outer nuclear membrane with the inner nuclear membrane at the peripheries of numerous pores, and the inner nuclear membrane with the nucleolar channel system. To fully understand the physiology of the cell, it is imperative to determine the composition of the system of channels or cavities--the extracellular space, the endoplasmic reticulum cavities, and the perinuclear cisternae. This study is concerned with the isolation of the nuclear envelopes and the determination of the composition of the perinuclear cisterna.

General nuclear envelope morphology. The nuclear envelope was first described by Callan and Tomlin (1950), who studied the oocytes of Triturus and Xenopus; it was described as double-layered with a continuous internal layer and a porous external layer. The nuclear envelope has since been a subject of investigation which has led to variable findings. Watson (1955) referred to the nuclear envelope as two nuclear membranes plus the perinuclear space separating the two membranes. Gall (1959) measured each nuclear membrane of oocytes of the newt

Triturus viridescens as 50-80 Å thick, a measurement which varies among authors: 90 Å (sea urchin and starfish oocytes, Afzelius, 1955), 100 Å (general tissues, Wischnitzer, 1960), 70-80 Å (mammalian tissues, Barnes and Davis, 1959), and 75 Å (amphibian oocytes, Wischnitzer, 1958). The generally accepted measurement is 75 Å, although it is understood that there is some variation from this value depending upon the tissue studied. Afzelius (1957) reported the appearance of dense particles approximately 150 Å in diameter on the outer surface of the outer membrane. These have been identified as ribosomes by Maggio, Siekevitz, and Palade (1963) in isolated nuclei.

Perinuclear cisternae morphology. The width of the perinuclear cisterna varies among tissues as many authors have reported: 200-400 Å (rat liver, spleen, pancreas, and stomach, Watson, 1955), 100-300 Å (rat liver, pancreas, and tongue, Watson, 1959), 150 Å (general tissues, Wischnitzer, 1960), 120-140 Å (mammalian tissue, Barnes and Davis, 1959), and 200-1000 Å (general tissues, Baud, 1959). The generally accepted dimension, subject to tissue variation, is 150 Å. The perinuclear cisterna is interrupted, usually uniformly, by pore complexes. The outer and inner nuclear membranes join at the periphery of the pore, the diameter of which is another subject of difference among authors: 250-750 Å (Watson, 1955), 400-1000 Å (Watson, 1959), 1000-1400 Å (Gall, 1959), 1000 Å (Wischnitzer, 1960), 500-600 Å (Gay, 1956), 700-1000 Å (Barnes

and Davis, 1959), 750-1000 Å (Wischnitzer, 1958), 300-500 Å (Baud, 1959), and 640 Å (Feldherr, 1965). The generally accepted measurement of the diameter is 750-1000 Å. On the other hand, Claude (1963), studying intact rat liver nuclei fixed in ion-free osmium tetroxide, could find no evidence of nuclear pores or annuli. Kodama (1969) did not observe any pores in isolated calf thymus nuclei fixed in osmium tetroxide, sodium permanganate, or acrolein. Several investigators have described rings or annuli which line the pore areas. The inside diameter of the annuli was measured as 400-500 Å by Watson (1955), as 970 Å by Afzelius (1955), and as 800 Å by Kautz and DeMarsh (1955). The annular wall measures 200-250 Å thick (Afzelius, 1955; Wischnitzer, 1958).

Annular morphology. The annuli were observed to extend a distance of 600 Å into the nucleus and 150-250 Å into the cytoplasm (Afzelius, 1955). Watson (1959) noted central granules within cross-sections of the pore complex. These appeared to be concentrations of organized material within the annuli at the level of the nuclear envelope. He described the annuli as the cortex of intranuclear channel (annular) material and of cytoplasmic channel extrusions. This has led some investigators to the idea that the nuclear envelope is actually a specialized cytoplasmic structure and that it is not properly part of the nucleus. Merriam (1961) determined that the composition of the annuli is a substance or substances which is partially fibrillar and is removable by trypsin

digestion, i.e., protein. Since the annulus remains with the nuclear envelope when it is isolated, Merriam (1961) concluded it is a part of the envelope's structure and not merely evidence of material passing through, as some authors have suggested. Gay (1956) described the annuli as granular with a dotlike structure in the center of the pore. Wischnitzer (1958) reported that at high magnification the annuli are resolved into subannuli, each 175-225 Å in diameter. Kautz and DeMarsh (1955) made densitometric tracings across the "light" center areas of the dense annuli and found them to be as dense or denser than the nuclear membrane outside the annuli. They interpreted this as meaning that the light areas are either not pores or are pores whose apertures are filled with some osmiophilic substance, the latter of which would support the diaphragm hypothesis.

Diaphragm. A very thin membrane at the waist of the pores at the level of the nuclear envelope has been termed the diaphragm (Watson, 1955; Watson, 1959; Merriam, 1961; Afzelius, 1955; Wischnitzer, 1960; Gay, 1956; Kautz and DeMarsh, 1955; Baud, 1959; Feldherr, 1965). Barnes and Davis (1959) suggested that the diaphragm does not exist but is only a part of the nuclear membrane surrounding the pores in a plane back of or in front of the section. Merriam (1961) determined that the diaphragm is removable by trypsin digestion or $KMnO_4$ fixation. Baud (1959) proposed that if the orientation of the nucleus is unfavorable or if the contrast

in the vicinity of the pore is weak, the diaphragm is not seen. Feldherr (1965) microinjected PVP-coated colloidal gold particles into the cytoplasm of Amoeba proteus and observed a very high concentration of gold along the nuclear envelope associated with the electron-opaque pore material (diaphragm). Even though the pore size was approximately 640 Å, the maximum size of gold particles able to enter the nucleus was from 125-145 Å. He concluded that the electron-opaque pore material fills a large portion of the pore area.

Perinuclear cisternae composition. The contents of the perinuclear cisterna have been described by Watson (1955) as having a different density than the cytoplasmic matrix. He, as well as others, Wischnitzer (1958) and Baud (1959), described the contents as clear and homogeneous. The chemical composition is not known (Baud, 1959). Palay (1960) traced particulate fat droplets from the lumen of the intestine in the rat and observed them within the perinuclear space of the nuclear envelope. He suggested that the contents of the perinuclear cisterna must therefore be fluid enough to allow the unobstructed passage of this particulate material. This would support the idea that the cisterna is a free water space. Baud (1959) described the thickness of the perinuclear cisternae as very irregular, depending on the course of the inner and outer nuclear membranes. He reported that some granulations have been seen in the perinuclear cisternae, and suggested that extensions of adjoining cells or nerve terminations may enter into the cisternae.

Relationship of endoplasmic reticulum and perinuclear cisterna. Several investigations have revealed a possible continuity between the perinuclear cisternae and endoplasmic reticulum. Palade (1955, 1956), Terzakis (1965) and Baud (1959) observed communications between the contents of the perinuclear cisterna and that of the endoplasmic reticulum. Palade (1955) suggested that the nuclear envelope is an extension of the endoplasmic reticulum and that pores may be related to fenestrae which have been identified within the intracytoplasmic cisternae of the reticulum. Watson (1955) described the nuclear membranes as appearing somewhat denser than the endoplasmic reticulum. Watson, studying cells of rat spleen, clearly demonstrated connections between the perinuclear cisterna and cavities of the endoplasmic reticulum. He also suggested that the outer and inner nuclear membranes appear as part of the endoplasmic reticulum. Palay (1960) in tracing particulate fat droplets from the lumen of the small intestine of the rat noted that the droplets could be located within the endoplasmic reticulum, within the Golgi Complex, and within the perinuclear cisterna. Palay suggested that the appearance of fat droplets within the perinuclear space constitutes evidence of physiological continuity between the endoplasmic reticulum and the nuclear envelope. Hadek and Swift (1962) studied trophoblast cells of a 6-day rabbit embryo and observed sections which frequently revealed continuities between the cisternal membranes of the endoplasmic

reticulum and the outer layer of the nuclear envelope. They observed dense inclusions within the perinuclear cisterna which were identical to inclusions observed within the cisternae of the endoplasmic reticulum. They suggested the inclusions represented nuclear extrusions through annular openings which are then distributed to the cisternae of the endoplasmic reticulum as they break off the nuclear envelope. Weston (1968), studying somite cells from 13-14 somite chick embryos, observed ribosome-like granules in polysome-like configurations in the perinuclear cisternae found within certain nuclear blebs which directly communicated with the lumens of tubules of the endoplasmic reticulum. Gay (1956), Watson (1955), Wischnitzer (1958) and Baud (1959) described the formation of these nuclear blebs as lens-shaped sacculations of the outer nuclear membrane projecting away from the inner nuclear membrane toward the cytoplasm. Gay (1956) described their formation following production of secretion granules in Drosophila. She suggested the blebs become flattened to form the endoplasmic reticulum. Swift (1956) suggested the formation of annulate lamellae which arise from the nuclear membrane, move out into the cytoplasm and break into numerous isolated cytoplasmic vesicles. Baud (1959) reported an increase in nuclear bleb formation when the nuclei are exposed to hypotonic solutions and also at the onset of autolysis. The perinuclear space within the blebs observed by Weston (1968) was separated from the remainder of the space by a very fine membrane. Terzakis (1965)

described a nucleolar channel system consisting of matrix, granules, and tubular channels which appeared to be in continuity with the perinuclear cisterna also.

Relationship of plasma membrane and perinuclear cisternae. Some authors even suggest that the perinuclear cisterna may be continuous with the extracellular fluid. Palade (1955, 1956) described a direct connection of the endoplasmic reticulum and the plasma membrane and suggested that the contents of the endoplasmic reticulum cavities communicate freely with the pericellular medium. McAlear and Edwards (1959), studying cells of the conidophore of the deutoromycete Stilbum zaccaloxanthum, observed continuities of the plasma membrane with the outer nuclear membrane. They also reported several interrelated endomembrane systems which may refer to the endoplasmic reticulum. Probably the most interesting suggestion of a continuity between the extracellular and intranuclear space is that of Langendorf, Siebert, and Nitz-Litzow (1964). They studied the sodium transfer processes in animal tissue using Na^{22} as a tracer, and found that the extracellular Na^{+} and intranuclear Na^{+} reached equilibrium within a very short time. They suggested that the rapid equilibration occurs by extracellular Na^{+} entering the nucleus through channel-like structures such as the endoplasmic reticulum and perinuclear cisterna which directly connect extracellular and intranuclear spaces. The nuclear concentration of Na^{+} was greater than that of the cytoplasm.

Although much of the above evidence suggests that the perinuclear cisterna may be a free-water space, no one has yet conclusively shown that it is so, and not composed of structural components such as lipid, protein, or bound colloidal material. Previously it was thought that the mitochondrial cristae were composed of a single reflected membrane which sandwiched a water space, the intracristal space, judging from osmium-fixed, sectioned mitochondria (Chandra, 1962). However, today from the works of Green (1959), Green and Fleischer (1962), Parsons (1963), and others, it is known that the "intracristal" space is not a water space, but is composed of solid material of lipid and structural protein. Another line of evidence which would lead one to suppose that the perinuclear cisterna is not a freely diffusible water space is that in many permeability studies the penetrant is never found in the perinuclear cisternae, e.g., Feldherr (1964, 1965) indicated that no gold particles had entered the perinuclear cisternae.

Nuclear isolation. Allfrey et al. (1952) contend that all nuclei isolated in aqueous media are ill-adapted for studies of enzyme-localization since extraction of water-soluble proteins occurs. But studies of the isolated nuclear membranes require an aqueous method of isolation, rather than a non-aqueous method, since organic solvents cause extraction of lipid components essential to the structure of the membranes. Simple saline media have sometimes been used, but more efficient separation of nuclei and mitochondria is possible with a sucrose medium (Schneider, 1948).

A sucrose medium alone causes clumping, distortion, and contamination of nuclear preparations (Schneider and Petermann, 1950). An ion is necessary to "harden" the nuclei without causing agglutination of the cytoplasm; $CaCl_2$ and $MgCl_2$ were found by Schneider and Petermann (1950) to be equally good for that purpose. Dauta-Mentré (1964) suggested the use of either Ca^{++} or K^{+} in nuclear preparations to maintain the integrity of the nuclei. Hogeboom, Schneider, and Striebich (1952), Gill (1965), Allfrey, Litau, and Mirsky (1964), and Anderson and Wilbur (1952) found that if $CaCl_2$ was used the result was a good yield of nuclei which were in excellent condition, and the preparations contained very few intact cells and mitochondria.

Membrane isolation methods. Neville (1960) developed a successful procedure for the isolation of plasma membranes. He found that the endoplasmic reticulum and mitochondrial membranes did not tear in the same fashion as the cell membranes but that they tended to remain as closed vesicles. Emmelot et al. (1964) modified Neville's procedure to produce purer fractions of cell membranes. Finean, Coleman, and Green (1966) used the modified procedure to isolate plasma membrane fractions from erythrocytes, epithelium, liver and kidney. They assessed the fractions for contamination of mitochondria and endoplasmic reticulum using structural, chemical and enzymological techniques. The epithelial plasma membrane fraction showed no appreciable contamination, but the preparations from

the kidney and liver showed a low percentage of contamination. No published reports were found which adapted this method for the isolation of nuclear membranes. This study, therefore, is the first example known of such an adaptation.

Fixation media. The use of potassium permanganate ($KMnO_4$) as a superior fixative and stain for electron microscopy of membrane systems was suggested by Luft (1956) and confirmed by Bradbury and Meek (1960). $KMnO_4$ enhances the tissue density and contrast, especially of membranes, often making further staining unnecessary. However, it has been found that $KMnO_4$-fixed materials are more difficult to section than OsO_4 (osmium tetroxide)-fixed materials (Luft, 1956). Glauert (1965) and Wischnitzer (1967) in their review articles concerning fixation media support the use of $KMnO_4$ for membrane systems. Gall (1959) fixed nuclear envelopes of the oocyte of the newt Triturus viridiscens with OsO_4 and $KMnO_4$ and observed that OsO_4-fixed envelopes revealed annular structures whereas $KMnO_4$-fixed material did not. He explained the differences in either of two ways: $KMnO_4$ preserves the membranes but dissolves the annuli and OsO_4 preserves both or, conversely, $KMnO_4$ preserves the envelopes as in life and OsO_4 reveals artifacts produced by shrinkage of material around the pores. Since annuli have been observed by several authors, the former explanation seems justified. Merriam (1961) confirmed the loss of annular material during $KMnO_4$ fixation and its preservation with OsO_4 fixation. But since the present study was not

concerned with annular structure, and good preservation and contrast of the membrane systems was desired, $KMnO_4$ is preferred.

Marker substances. The mechanism of ingestion and transport of colloidal particles by cells or nuclei can be studied using electron dense particles such as ferritin or colloidal gold (Glauert, 1965) or horseradish peroxidase (Strauss, 1964). Horseradish peroxidase has been used as a marker substance in several investigations, primarily to trace fluid, solute, or protein transfer within cells or tubules of different tissues (Graham and Karnovsky, 1966; Cotran and Karnovsky, 1968; Eisenberg and Eisenberg, 1968; Strauss, 1964). Cotran and Karnovsky (1968) found that the peroxidase entered the cells before, during and after fixation. Some of the peroxidase could be washed away but binding had occurred.

Feldherr (1964, 1965) used colloidal gold to study nuclear permeability in Amoeba proteus and oocytes of Rana pipiens. He coated the gold particles with either polyvinylpyrrolidone (PVP), poly-l-proline or poly-l-lysine to dismiss variations in distribution which could be attributed to chemical differences. He exposed both intact and ruptured nuclei to the gold particles and observed high concentrations of gold binding to the pore material. Feldherr (1964) suggested the pore material could be responsible for selecting and accumulating substances to be transported. He also suggested that the nuclear pore was positively charged since he found that

negatively-charged coated gold particles were bound to the pores while positively-charged particles were located farther from the pores. Risueño, Giménez-Martín, and López-Sáez (1965), studying the nuclei of seeds of Phalaris canariensis, also suggested the idea of a positively-charged nuclear membrane. He found definite localizations of negatively-charged chromatin near invaginations of the nuclear membrane whereas the positively-charged nuclear sap seemed to repel the nuclear membrane, causing scalloping of the otherwise spherical nuclear envelope.

Ferritin. As described by Glauert (1965) and Farrant (1954), ferritin is a protein containing 20% iron, in the form of ferric hydroxide-phosphate, with a protein shell. The protein shell, apo-ferritin, has a molecular weight of about 460,000 and is nearly spherical with a diameter of approximately 110 Å. The ferric hydroxide micelles, within the protein shell, are arranged at the apices of a roughly tetrahedral lattice measuring about 55 Å across. The molecular diameter of ferritin by electron microscopy has been determined at 94 ± 5 Å.

Huxley (1964), Page (1964), and Peachey and Schild (1968) used ferritin as a marker protein in testing the continuity of the extracellular space with the T-system of the frog sartorius muscle. Page (1964) and Huxley (1964) exposed the muscle tissue to ferritin before and during fixation and found that the ferritin had entered the T-system. However,

when muscle tissue that had been fixed for two hours was exposed to ferritin, no ferritin was found to enter the T-system.

Ferritin is easy to recognize under the electron microscope without staining (Huxley, 1964) and has a molecular size (100 Å) which is less than the width of the perinuclear cisterna. Although it has been reported by Casely-Smith (1962) that there is some displacement of ferritin during sectioning, the amount displaced is small relative to the total amount of ferritin present and therefore, it was not considered a problem in this study.

Phosphotungstate. The use of phosphotungstate (PTA) as a marker was also attempted in this study. Brenner and Horne (1959) introduced PTA as an effective negative stain for electron microscopy in that it provides good preservation and high contrast. Parsons (1963) and Fernandéz-Morán (1962) used PTA as a negative stain for mitochondrial membranes and produced high contrast to characterize the structural components of the mitochondrial membranes. Prezbindowski, Sun, and Crane (1968) used PTA to study the Golgi system, lysosomes, and mitochondria.

The present study, however, employed PTA with the assumption that not all of the PTA crystals would dissolve and would therefore serve as markers. The staining properties of PTA were not desired.

Embedding media. Epoxy resins provide remarkable freedom from polymerization damage with consequent excellent

preservation of cellular fine structure and intercellular relationships (Luft, 1961). Epoxy resin-embedded sections have been observed to suffer less degradation than sections embedded in other media during irradiation by the electron beam. No supporting film on the grid is necessary. Epon 812 was the epoxy resin chosen for this study, based on the recommendation of Luft (1961).

Purpose of study. This study was undertaken to isolate mass quantities of nuclear envelopes. Such an isolation of relatively pure nuclear envelopes would facilitate biochemical studies on the composition, enzymology, permeability, and physical characteristics of the envelope. In the process of isolating the membranes, a method for aqueously isolating nuclei was refined. In addition to the isolation, it was attempted to determine whether the perinuclear cisterna is an integral part of the nuclear membrane barrier complex. The equilibration of marker substances (PTA crystals and ferritin) within the perinuclear space would suggest a water content freely available for diffusion of solutes and colloidal particles, whereas their exclusion would suggest a relatively non-diffusible bound-water space or lipid content.

MATERIALS AND METHODS

Trial variations. The general methods of Chaveau, Moulé, and Rouiller (1956) were used for the preparation and isolation of fresh, rat liver nuclei, but several modifications

were made on the basis of preliminary studies. Blood was eliminated in the liver by perfusion in situ with the principal isolation medium to be referred to in this study as Solution I (0.25 osmol sucrose [Fisher Scientific Products], 0.02 M Tris, 0.003 M $CaCl_2$, pH adjusted to 7.4 at room temperature) through the superior vena cava and through the hepatic portal system. Perfusion through the portal system proved more effective, producing a marked change in the color of the liver from dark brown to light beige, and so was the method of perfusion chosen.

The Potter-Elvehjem (P-E) Homogenizer was used to disrupt the whole liver cells. A glass mortar and tight, rotating, teflon pestle were used. In a preliminary trial, two portions of minced tissue were homogenized, one portion with three strokes and the other with twenty strokes, and the resulting homogenates were compared under the light microscope. The three-stroke homogenate contained many whole cells and relatively few free nuclei. The twenty-stroke homogenate contained more free nuclei and fewer whole cells and therefore seemed more effective. All nuclear isolation homogenizations were made with twenty strokes with the tight pestle.

In the initial trials, the crude homogenate was centrifuged at 1,000 x g for 15 min. However, the pellet became packed too tightly and remained in clumps when resuspended. Centrifugation at 600 x g for 5 min was found to be preferable since it produced a loose pellet of comparable yield and

prevented clumping. In determining the density gradient to be used, several trial runs were made. The following concentrations of sucrose were found too dense to allow sedimentation of the nuclear fraction: 2.2 M, 2.0 M, 1.9 M. The following solution of sucrose had the most effective density: 1.8 M sucrose, 0.02 M Tris, 0.003 M $CaCl_2$, pH adjusted to 7.4 at room temperature. An attempt was made to duplicate the nuclear isolation procedure of Maggio et al. (1963) of layering 0.88 M sucrose over 2.2 M sucrose for guinea pig nuclear isolation, but the yield was too small for further experimentation.

Nuclear isolation. The isolation procedure used was a modification of the procedure of Chaveau et al. (1956) as has been indicated. Unfasted rats of the Sasco Strain, weighing approximately 300 g, were killed by cervical dislocation and were bled by decapitation. The liver was perfused with Solution I at 0-4°C through the hepatic portal vein and excised. Individual lobes were perfused when necessary. All subsequent steps of nuclear isolation were carried out at 0-4°C. The liver lobes were minced on ice with a scissors. The minced tissue (approximately 12 ml) was diluted 1:4 (1 part minced tissue:4 parts total volume) with Solution I and homogenized twenty strokes with the P-E Homogenizer. The homogenate was pooled and diluted 1:5 with Solution I to a final volume of 250 ml. The dilute homogenate was strained through a double layer of nylon mesh and centrifuged at 600 x g for 5 min in the International Refrigerated Centrifuge, Model B-20, Rotor

No. 870, set at -2°C. The supernatant fluid, except for a sample, was discarded, and the pellets were pooled and washed by resuspension in Solution I up to the previous volume (250 ml). The suspension was again centrifuged at 600 x g for 5 min. The sample of supernatant fluid was observed under the light microscope to contain small subcellular particles, commonly identified as mitochondria, lysosomes, microsomes, and glycogen granules (Schneider and Hogeboom, 1951; Luck, 1961). The washing procedure was repeated two more times to remove the smaller subcellular particles. The pellets (8 ml approximate total volume) of the last centrifugation were resuspended in Solution I up to a volume of 160 ml. In each of eight 50 ml polyethylene centrifuge tubes 20 ml of the suspension were layered over 20 ml of 1.8 M sucrose containing 0.003 M $CaCl_2$ and 0.02 M Tris, with a J-shape needle on a 20 ml glass syringe. The tubes were centrifuged at 45,000 x g for one hour. The interface particles were removed with a syringe for examination under the light microscope, and the remaining supernatant fluid was discarded. The interface particles were found to consist primarily of whole cells, cytoplasmic debris, erythrocytes, mitochondria, and some nuclei, as Maggio et al. (1963) had found in their similar nuclear isolation procedure. The nuclear pellets, which varied in amount in different experiments (usually about 5-10% of original homogenate), were pooled and resuspended in Solution I to a volume of 160 ml, stirred well, and centrifuged at 600 x g for 5 min. The supernatant fluid

was discarded and the pellets were pooled and resuspended in 0.003 M $CaCl_2$ up to a volume of 160 ml. This suspension was centrifuged at 600 x g for 5 min. The resultant pellets were resuspended in 0.003 M $CaCl_2$ up to a volume of 8 ml. At this point homogeneous samples of the suspension were viewed under a bright field light microscope and photographed. The resultant light micrographs of the nuclear preparation were analyzed for whole cell contamination and non-nuclear volume per cent contamination. Counts were made on micrographs of six different fields of view. The whole cells and nuclei per micrograph were counted and the percentage of whole cells per total number of nuclei plus whole cells (whole cells/nuclei plus whole cells) was calculated. The mean average of the values for whole cell contamination was determined. The non-nuclear volume per cent contamination was determined by crudely estimating the total per cent of the volume of material shown on each micrograph which was non-nuclear material. Therefore, the remaining percentage of volume was nuclear material. A mean average for these estimates was determined.

Nuclear envelope isolation. The nuclear envelope isolation procedure used was a modification of Neville's (1960) procedure for isolation of cell membranes. The 8 ml nuclear suspension was homogenized 50 strokes with the P-E Homogenizer. The mortar was immersed in ice water during the homogenization to prevent an excessive temperature rise in the homogenate. All subsequent steps of nuclear envelope isolation were carried out at room temperature.

Nine milliliters of 37% sucrose were layered over a mixture of 6.2 ml of the homogenate and 11.6 ml of 69% sucrose in a 50 ml centrifuge tube, with a J-shape needle on a glass syringe. The tube was centrifuged two hours at 45,000 x g. The remaining suspension was observed under the light microscope and photographed.

Three separate fractions were observed and photographed under the light microscope: Fraction 1 was a white fluffy layer at the upper half of the interface; Fraction 2, a dark tan layer at the lower half of the interface; and Fraction 3, a pellet. Fraction 1 appeared to be the nuclear envelope fraction and was washed twice by resuspending in 0.003 M $CaCl_2$ and centrifuging at 1,720 x g for 15 min. All of the supernatant fluid was removed with a pipette.

Electron microscopy. One-half of the 0.25 ml pellet of fractionated nuclear envelopes was exposed to 0.1% PTA and the other half to 1.3% Horse Spleen Ferritin. The fixation medium used was $KMnO_4$ as recommended by Luft (1956). To one-half of the pellet was added 0.2% PTA (Ladd Research Industries) (0.2 g/100 ml 0.003 M $CaCl_2$) up to a volume of 3 ml and 2.0% $KMnO_4$ (Ladd Research Industries) (2 g/100 ml 0.003 M $CaCl_2$) up to a final volume of 6 ml, giving a final concentration of 0.1% PTA and 1.0% $KMnO_4$. To the other half were added 0.003 M $CaCl_2$ up to a volume of 2 ml, 1 ml of 10.4% ferritin (Polysciences, Inc.) and 3 ml of 2.0% $KMnO_4$ (total volume of 6 ml), giving a final concentration of 1.0% $KMnO_4$ and 1.3%

ferritin. The tubes were shaken well and the fixation was allowed to proceed in the refrigerator (approximately 8°C).

The tubes were centrifuged in an International Clinical Centrifuge set at 1,720 x g for 15 min. The supernatant fluid of each tube was poured into each of two other 15 ml conical centrifuge tubes to recover any unsedimented membranes. The pellet of each of the two original tubes was resuspended in distilled water up to a volume of approximately 6 ml. The four tubes were centrifuged at 1,720 x g for 15 min. The supernatant fluid was removed with a pipette, and each pellet was washed again. The PTA-treated pellets and the ferritin-treated pellets were each resuspended in distilled water, pooled, and centrifuged at 1,720 x g for 15 min. The supernatant fluid was removed with a pipette, and each of the two pellets was resuspended in 8 ml of distilled water and homogenized ten strokes with the P-E Homogenizer, and centrifuged at 1,720 x g for 15 min. The supernatant fluids were discarded. One milliliter of 10.4% ferritin and 7 ml of distilled water were added to the ferritin-treated pellet. To the PTA-treated pellet were added 8 ml of 0.1% PTA (0.1 g/100 ml distilled water). The tubes were shaken well and centrifuged at 1,720 x g for 15 min. All of the supernatant fluid in each tube was removed with a pipette.

The pellets were thoroughly suspended in 2% agar according to the methods of Kellenberger, Ryter, and Séchaud (1958) and Kodama and Tedeschi (1963). Drops of the agar-suspended

membranes were placed on a cooled microscope slide. The hardened agar was cut into 1 mm cubes. A few of the cubes were placed in distilled water and refrigerated until photographed. The remaining agar cubes were processed for electron microscopy. The cubes were dehydrated in gradations of ethanol, remaining 15 min each in the following: 50%, 75%, 95%, 100% (twice) alcohol solutions and twice in pure propylene oxide (Ladd Research Industries).

The embedding medium used was Epon 812 (Ladd Research Industries), according to Luft (1961). The complete resin mixture, resin components plus DMP-30 (accelerator), was prepared in the ratio of MNA(methyl nadic anhydride):DDSA(dodecenyl succinic anhydride) of 7:3.

The infiltration and embedding procedure used was a modification of Luft's procedure (1961). The agar cubes were transferred from the infiltrating medium into a vial of pure Epon, stirred thoroughly and allowed to stand at room temperature for 4 hr to insure complete infiltration. The cubes were embedded in gelatin capsules, placed in a 60°C oven and allowed to polymerize for 48 hr. The blocks were trimmed, and sectioned with a glass knife on a Sorvall-Porter Blum MT-1 Microtome. The sections (ranging from 500A to 1000 A) were mounted on bare, washed, 400 mesh copper grids for observation on a Hitachi HS7S Electron Microscope.

RESULTS

Light microscopy. Figures 1-3 show isolated free nuclei which appear to be in excellent condition. Whole cell contamination of the nuclear preparation is shown in Table 1 and non-nuclear volume per cent contamination is shown in Table 2. The average whole cell contamination of the nuclear preparation was 1.0%. The average non-nuclear volume per cent contamination (crudely estimated) was approximately 4%. Therefore, the average volume per cent of nuclei per field of view was 96%. Since the erythrocytes cannot be distinguished from the nuclei on micrographs, estimates of erythrocyte contamination were made while the preparations were studied under the light microscope. The estimated erythrocyte contamination was less than 0.5%.

The nuclear preparation after homogenization is shown in Figures 4 and 5. In Figure 4, a typical field of view, it is estimated that there were twice as many disrupted nuclei as there were whole nuclei. Therefore, approximately two-thirds of the original whole nuclei were disrupted.

As a part of membrane isolation the three separated fractions were observed and photographed. Fraction 1 (Figures 6-9) appears to be a pure membrane fraction. The membranes, however, are accompanied by clumped nucleoplasm with some possible cytoplasmic material. Some of the apparent contamination may be other membranes, such as mitochondria, plasma membranes, and endoplasmic reticulum, but the large bulk of it

TABLE 1. Nuclear Preparation Whole Cell Contamination

Field of View	No. Whole Cells	No. Nuclei + Whole Cells	% Whole Cell Contamination
1	1	61	1.6%
2	0	27	0.0%
3	0	82	0.0%
4	1	114	0.9%
5	1	57	1.8%
6	1	45	2.2%
Overall Average Contamination:	4	386	1.0%

TABLE 2. Nuclear Preparation Per Cent Volume Contamination

Field of View	% Vol. Nuclei	% Vol. Non-nuclear Material
1	93%	7%
2	99%	1%
3	96%	4%
4	97%	3%
5	98%	2%
6	96%	4%
Overall Average % Volume Contamination		3.5%

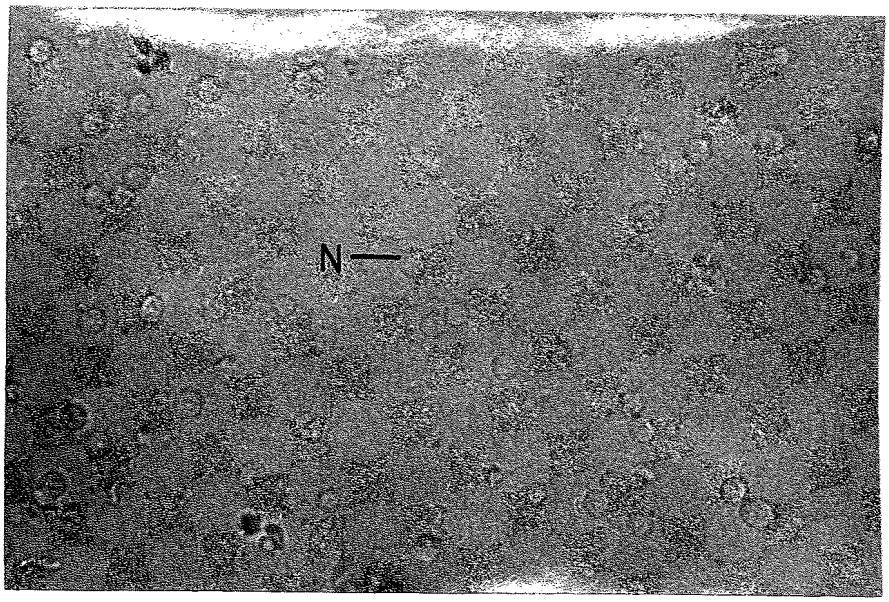

Figure 1. Typical light micrograph showing relatively pure rat liver nuclear preparation. Nuclei (N) appear to be in excellent condition. Magnification = 400x

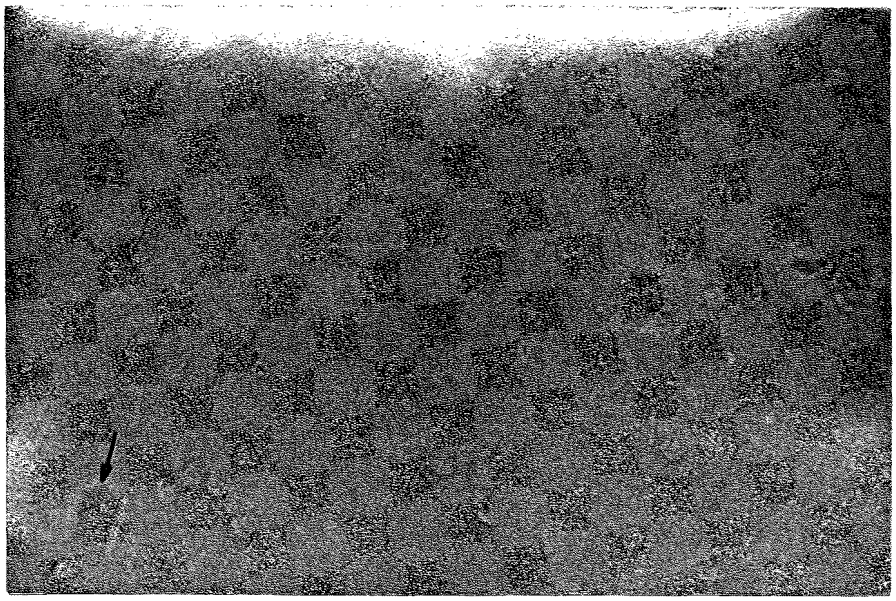

Figure 2. Light micrograph showing relatively pure nuclear preparation in a different field of view. One whole cell is seen (arrow). Magnification = 400x

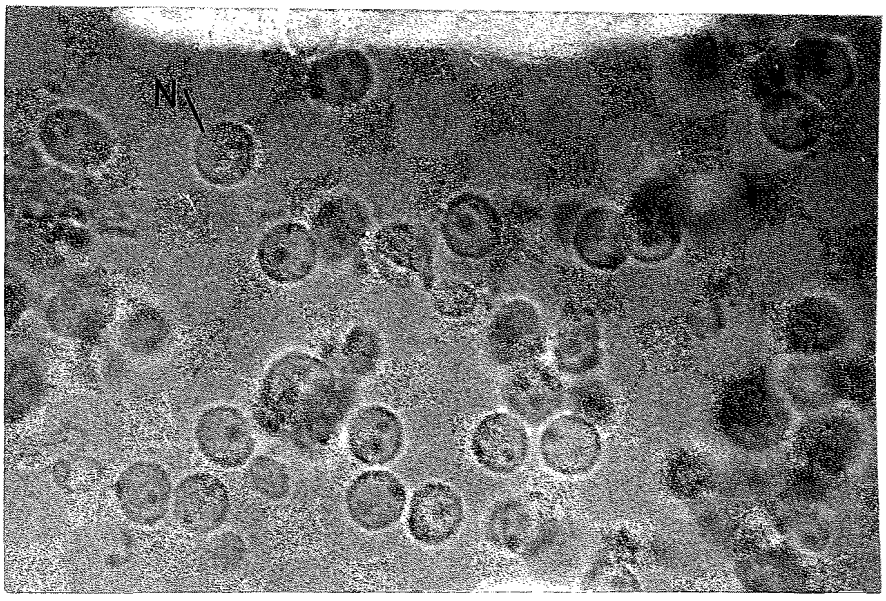

Figure 3. Light micrograph showing nuclear preparation under oil immersion. Nuclei (N) appear normal, containing one or two nucleoli. Magnification = 910x

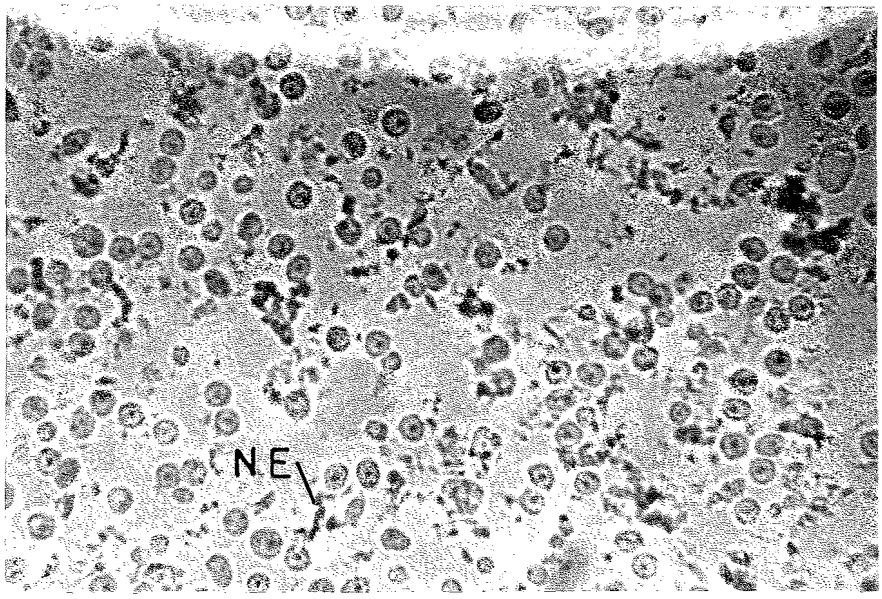

Figure 4. Light micrograph showing nuclear preparation after homogenization. Note the nuclear envelope fragments (NE). Magnification = 400x

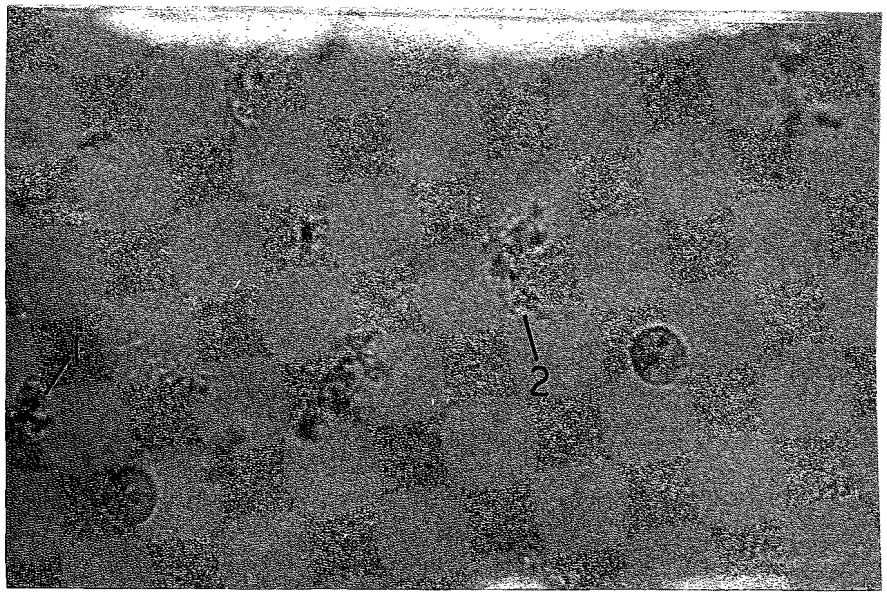

Figure 5. Light micrograph showing nuclear preparation after homogenization under oil immersion. Note misshapen nucleus (1) and nuclear envelope fragments (2). Magnification = 970x

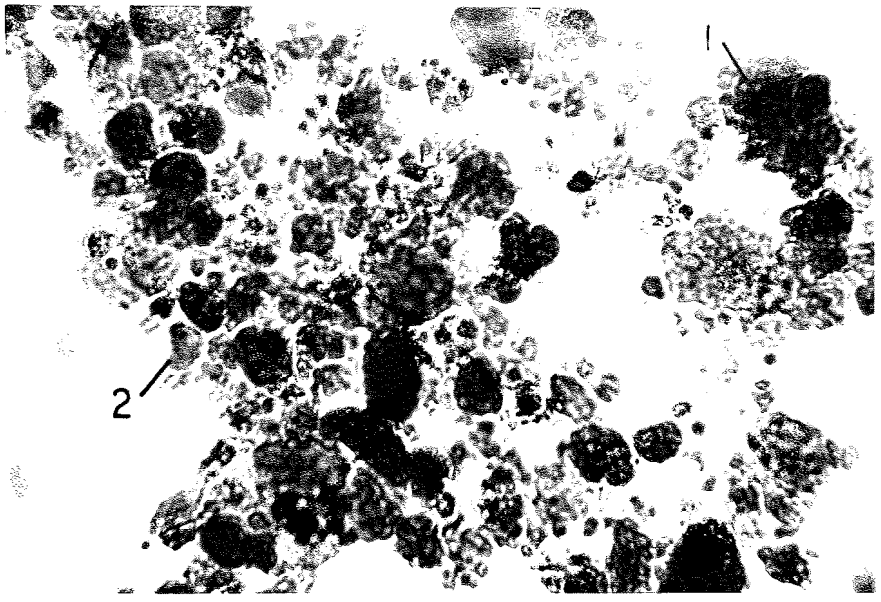

Figure 6. Light micrograph showing Fraction 1, fixed with $KMnO_4$ and exposed to ferritin. Note flattened nuclear envelope (1) and nuclear envelopes curled upon themselves (2). Out-of-focus figures are contaminants in the optical lens system. Magnification = 970x

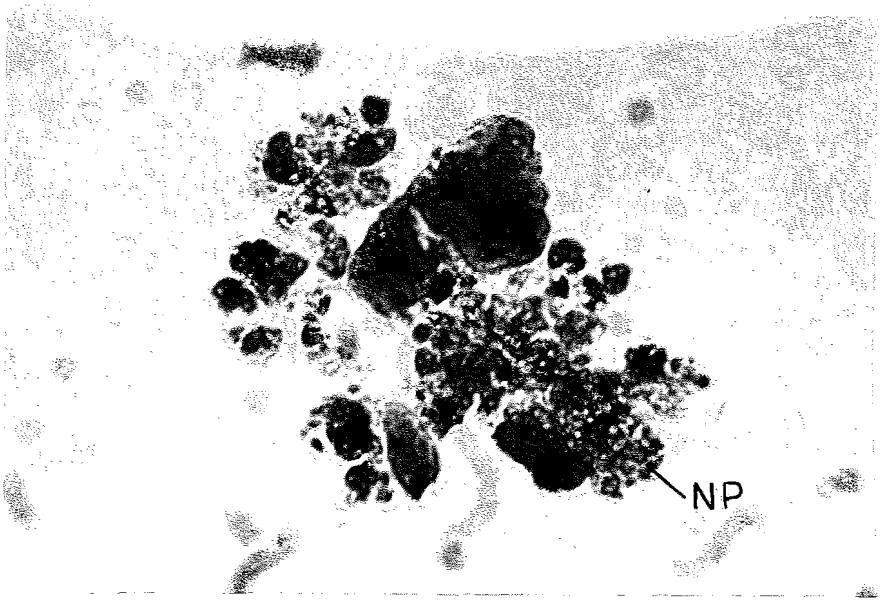

Figure 7. Light micrograph showing Fraction 1, fixed with $KMnO_4$ and exposed to ferritin. Note nucleoplasm adhering to nuclear envelopes (NP). Out-of-focus figures are contaminants in the optical lens system. Magnification = 970x

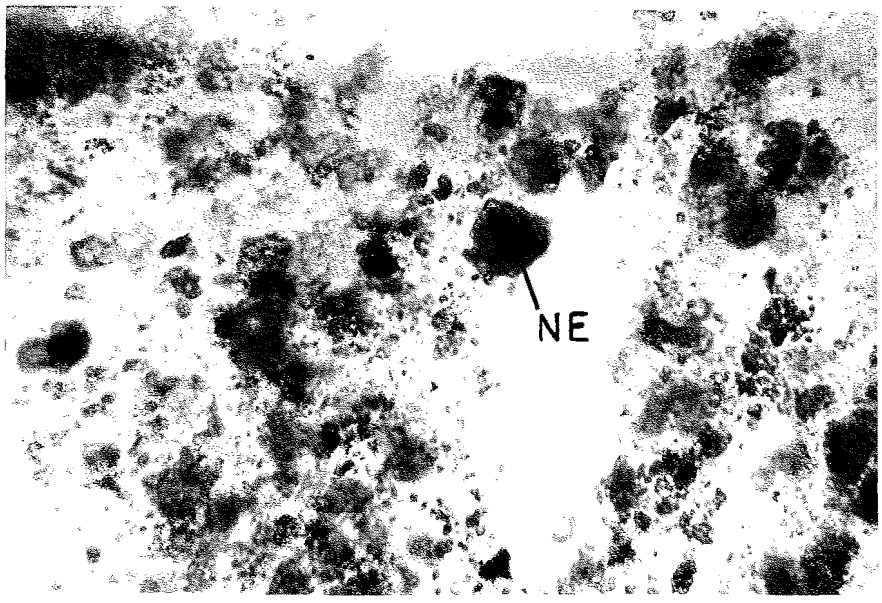

Figure 8. Light micrograph showing Fraction 1 fixed with $KMnO_4$ and exposed to PTA. Note nuclear envelope fragments (NE). Magnification = 370x

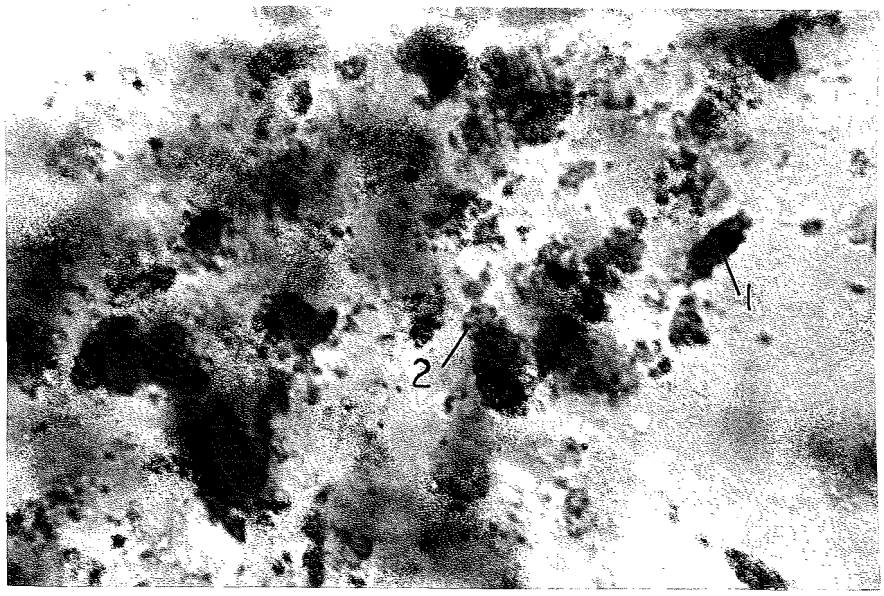

Figure 9. Light micrograph showing Fraction 1 fixed with $KMnO_4$ and exposed to PTA. Note nuclear envelope fragments (1) with adhering nucleoplasm (2). Magnification = 890x.

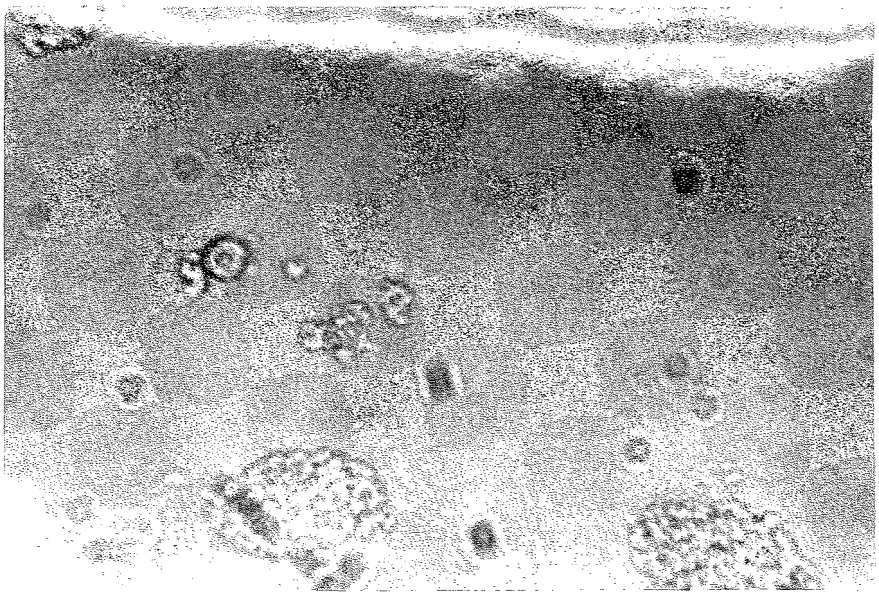

Figure 10. Light micrograph showing Fraction 2, unfixed. Note clumps of nucleoplasm with some possible cytoplasmic material. Out-of-focus figures are contaminants in the optical lens system. Magnification = 970x

is amorphous material. Since the nuclear preparation was relatively pure, it is safe to assume very little contamination by other membranes. The nuclear envelope fragments in Figures 6-9 appear to have nucleoplasm adhering to them. Some of the fragments are flattened and others have curled upon themselves.

Fraction 2 (Figure 10) appears to consist of clusters of amorphous substance, possibly with entrapped mitochondria. Very few free nuclei were seen. Although indistinguishable, nuclear envelopes may be a part of the clusters of debris.

Fraction 3 (Figures 11 and 12) is a pure nuclear fraction, representing the whole, intact nuclei shown in Figures 4 and 5. Several nuclei are misshapen but they remain intact. Very few nuclear envelope fragments can be seen.

Electron microscopy. The nuclear envelope preparation exposed to 0.1% PTA did not reveal PTA crystals in the photographs as was desired (Figures 13 and 14). The membranes shown on the electron micrographs are probably unit membrane structures since they are about 70-100 Å thick, the average thickness of a unit membrane. The trilaminar structure of the unit membrane is easily distinguished in Figures 13, 14, 15, and 17. Figure 13 reveals some possible displacement of membrane material by the glass knife during sectioning, which is probable since Casely-Smith (1962) reported the displacement of ferritin during sectioning. In Figure 15 the trilaminar structure of the membranes can definitely be seen, but in portions only granules can be seen and at these points the structure

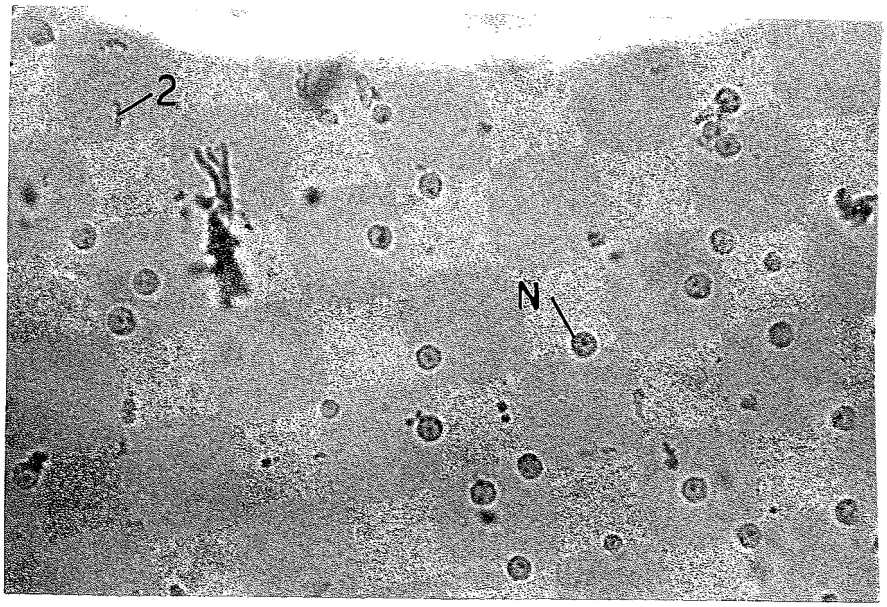

Figure 11. Light micrograph showing Fraction 3, unfixed. Note free nuclei (N) and very few membrane fragments (2). Magnification = 420x

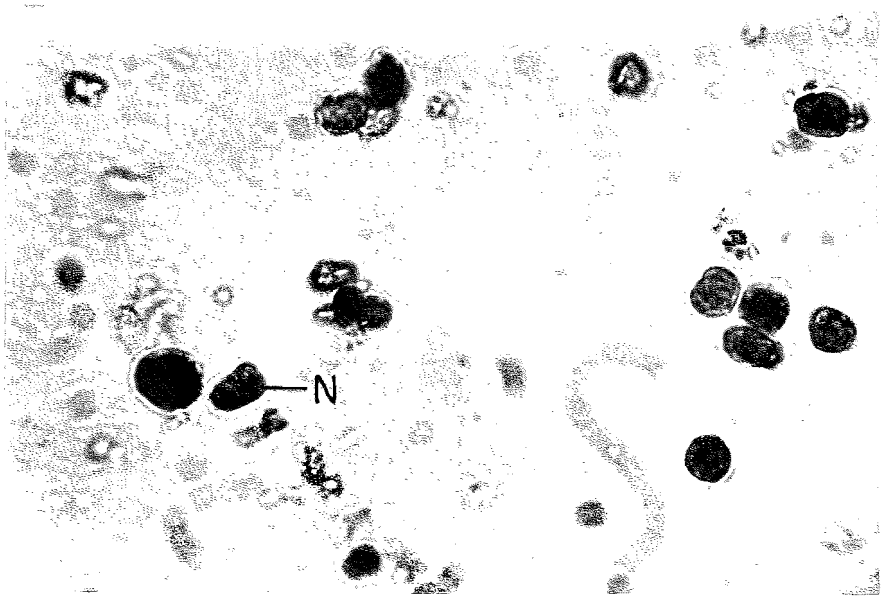

Figure 12. Light micrograph showing Fraction 3 fixed with $KMnO_4$ and exposed to ferritin. Note free, misshapen nuclei (N). Out-of-focus figures are contaminants in the optical lens system. Magnification = 970x

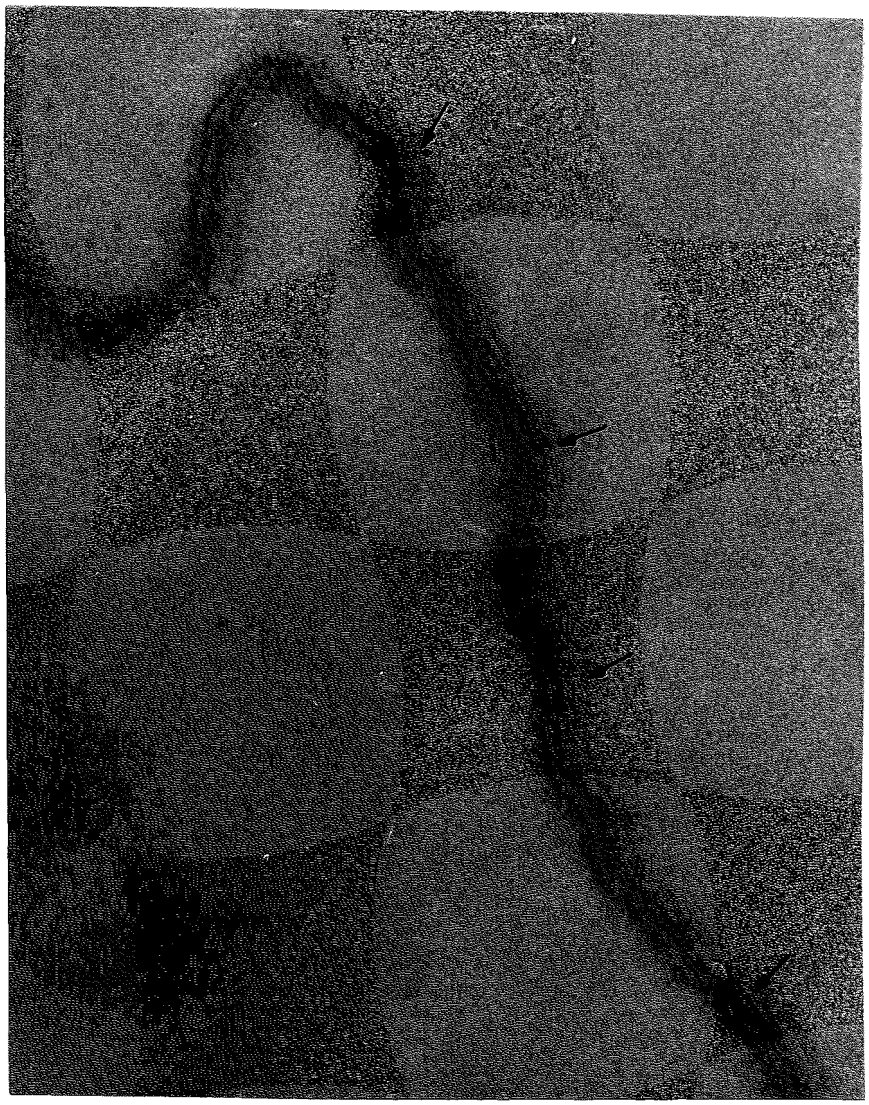

Figure 13. Typical electron micrograph showing a single trilaminar unit membrane structure with possible displacement of membrane material (arrows) by the knife during sectioning. This membrane was exposed to PTA but no crystals are seen. Magnification = 186,000x

Figure 14. Typical electron micrograph showing single trilaminar unit membrane structure. This membrane was exposed to PTA but no crystals are seen. Black spots (arrows) indicate holes seen in negative. Magnification = 186,000x

Figure 15. Typical electron micrograph showing ferritin-exposed isolated nuclear membranes sectioned normal to the plane of many membranes. The trilaminar structures of the individual unit membranes measure about 70 Å in thickness. Although many of the outer and inner nuclear membranes are separated, some appear to be paired, forming the perinuclear cisterna (arrows) between them, as in the intact nucleus. The ferritin particles can be seen as very dense granules (approximately 35 Å) closely adherent to both sides of all the individual membranes. Magnification = 143,000x

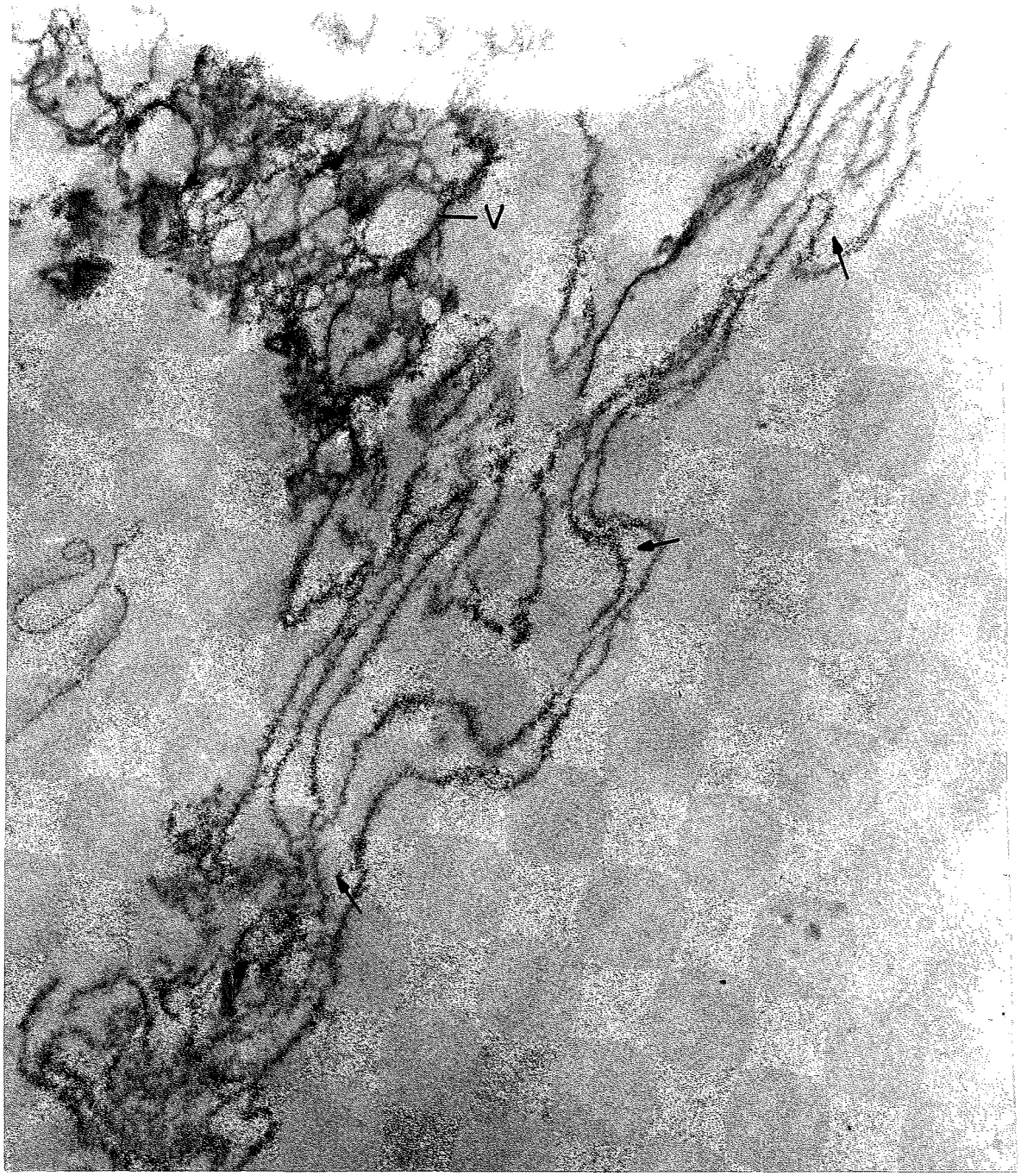

Figure 16. Typical electron micrograph showing ferritin-exposed isolated nuclear membranes. A pair of nuclear membranes appears to form the perinuclear cisterna (arrows) between them. The perinuclear cisterna ranges from 100 Å to 450 Å wide. Individual membranes measure approximately 100 Å. Ferritin is seen adhering on both sides of all the individual membranes. A group of re-formed vesicles of membrane fragments (V) can be seen. Magnification = 47,000x

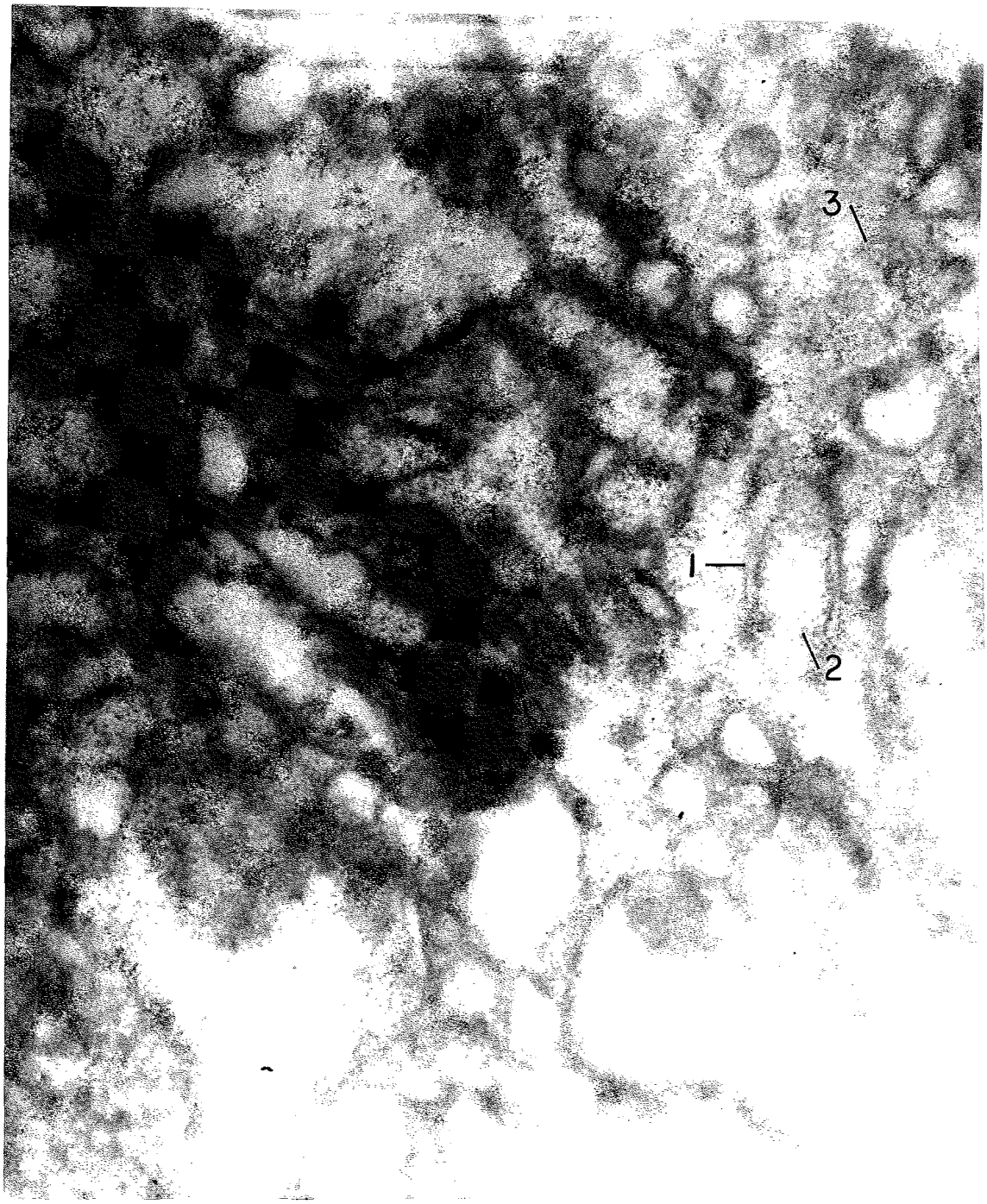

Figure 17. Typical electron micrograph showing ferritin-exposed membrane fragments re-formed as vesicles. A small portion of a possible nuclear envelope (1) with ferritin adhering to both sides of each of the individual nuclear membranes is seen. Ferritin (2) can also be seen within the perinuclear cisterna. A possible mitochondrial membrane is indicated (3). Magnification = 186,000x

appears much thicker. For this there are two possible explanations: (1) the plane of sectioning is parallel to the membrane (2) a sectioning artifact is present--displacement of material by the knife during sectioning.

The membranes in Figures 15-19 are those which were exposed to ferritin. Figure 17 clearly reveals the individual ferritin particles. The granules (35 Å) are of approximately the same order of magnitude and size as ferritin (55 Å) (Glauert, 1965; Farrant, 1954), and they are uniform in size in any one photograph. If the granules were nuclear or cytoplasmic granules they would show a wider variation in size. The size of the ferritin particle can vary due to a number of factors, e.g., focus, and stainability of the protein shell by $KMnO_4$. All of the micrographs (Figures 15-19) show ferritin on both sides of all of the individual unit membranes, even those which seem to have their cisternae intact. The ferritin particles were negatively charged at the pH of the solutions of this study.

Figures 16, 17, and 18 reveal vesicular structures which appear to be re-formed vesicles of smaller membrane fragments. The unit membrane structure is easily distinguishable on portions of the membranes in Figure 15. It is evident from Figures 15, 16, 18, and 19 that some of the unit membranes have separated from each other while others remain paired, strengthening their identification as isolated nuclear envelopes.

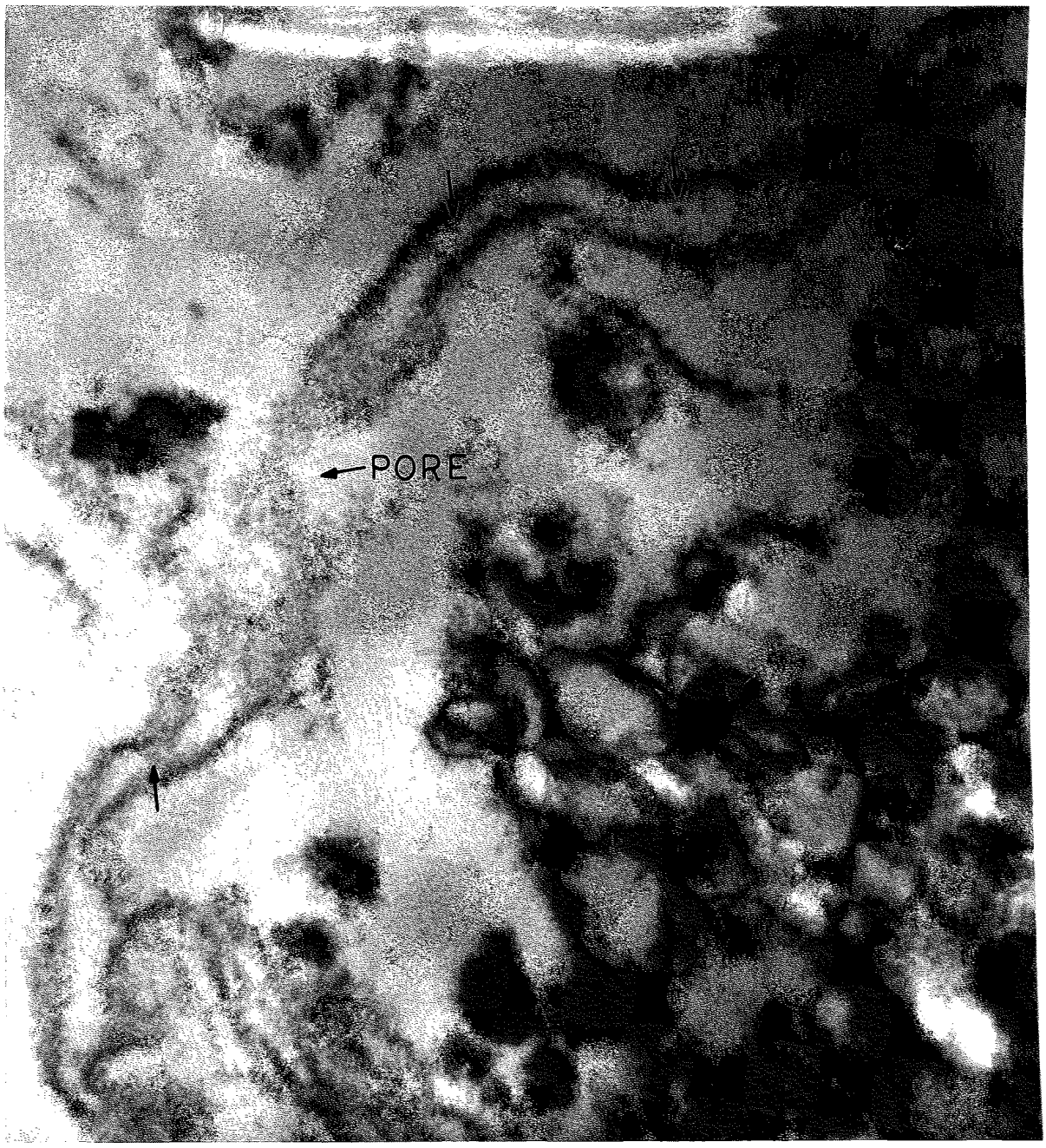

Figure 18. Typical electron micrograph, not perfectly focused, showing ferritin-exposed isolated nuclear membranes. A nuclear envelope appears to contain a nuclear pore area (1200 A wide). Ferritin particles (arrows) appear within the perinuclear cisterna as well as adherent to the outside of the membranes. Although clearly out of focus, re-formed vesicles of membrane fragments are seen. Magnification = 75,900x

Figure 19. Typical electron micrograph, not perfectly focused, showing ferritin-exposed isolated nuclear membranes. A nuclear envelope appears to contain a nuclear pore area (P) (880 Å wide) where inner and outer nuclear membranes have joined. The pair of nuclear membranes (arrows) seems to have separated on one side of the pore area. Magnification = 60,500x

In Figures 18 and 19 are nuclear envelopes with possible nuclear pore areas. Figure 18 shows the ferritin particles within the perinuclear cisternae as well as adherent to both sides of each of the membranes. Although the membranes in Figures 18 and 19 seem too thick to be unit membranes, the photographs were not perfectly focused, and the fine detail of the membranes cannot be seen. It is probable that what is seen are membranes plus adherent ferritin or nucleoplasmic granules which give added thickness to the membranes. Due to the poor resolution in the photographs, the granules cannot be distinguished from the membrane proper. When a specimen is out of focus it appears larger because the diffraction lines at the borders are dark and are interpreted as part of the structure. On the other hand, the other micrographs, properly focused at higher magnifications, all have membranes within the 70-100 Å range, and in these the expected trilaminar structure of the unit membrane can be positively identified (Figures 13, 14, and 15).

DISCUSSION

The isolated nuclear envelope fraction (Fraction 1) appeared to be pure as shown in the light micrographs and electron micrographs. The presence of nucleoplasm and cytoplasmic ground substance cannot be considered "contamination" as such since they would not contribute contaminating membranes. The possibility that a few mitochondria remain in the nuclear

envelope fraction cannot be eliminated, but such membrane contamination should be easily distinguishable in electron micrographs because of the presence of cristae in mitochondria and because mitochondrial membranes have shorter lengths than the nuclear membranes.

The following consideration of the methods for isolation of the nuclei further supports the contention that significant numbers of membranes other than nuclear membranes are not part of the membrane fraction. The crude liver homogenate was washed four times with Solution I by centrifuging at 600 x g for 5 min; the supernatant fluid was discarded after each washing. This centrifugation of the crude homogenate allowed sedimentation of the nuclei, unbroken liver cells, and red blood cells, known to occur at the centrifugal force of 600-800 x g. Sedimentation of mitochondria requires a centrifugal force of at least 5,000 x g; sedimentation of endoplasmic reticulum and microsomes requires a centrifugal force from 41,000 x g to 141,000 x g (Schneider, 1948; Hogeboom, Schneider and Palade, 1948; Schneider and Petermann, 1950; Schneider and Hogeboom, 1951; Siekevitz and Palade, 1958; Allfrey, 1959). Therefore, it is unreasonable to predict the presence of significant numbers of contaminating mitochondrial and endoplasmic reticulum membranes in the nuclear preparation. The prolonged centrifugation of the nuclear suspension at the high centrifugal force of 45,000 x g removed whole cells and red blood cells by retaining them at the interface and allowed

only nuclei to sediment through the density gradient. The purity of the nuclear preparation shown in the light micrographs (Figures 1-3) supports this contention. The purity of Fraction 1 is further supported by examining the components of Fractions 2 and 3 which were separated from the nuclear envelope fraction in the nuclear envelope isolation procedure. Fraction 2 contains amorphous material, probably nucleoplasm with some possible cytoplasmic material or a few entrapped mitochondria, nuclei, and membrane fragments which cannot be distinguished. Accurate descriptions are not possible without further assay studies of this fraction. The light micrograph of this fraction is the only means of identification in this study. Fraction 3 contained a pure nuclear fraction, the free intact nuclei that remained after homogenization of the nuclear preparation. With the cytoplasmic debris, nucleoplasm, and whole nuclei removed, the nuclear envelope fraction was purified. It is therefore concluded that this study succeeded in adapting the procedure of Neville (1960) for the isolation of plasma membranes to the isolation of nuclear envelopes.

As can be seen in Figures 13 and 14, PTA crystals did not serve as markers as was desired. The PTA mixture was prepared in a low concentration to prevent complete darkening of the background but was apparently too low to allow the PTA crystals to remain in suspension. All of the PTA crystals must have dissolved since no crystals were seen on any of the grids scanned under the electron microscope.

The trilaminar unit membrane structures have been indicated on the electron micrographs in the results section. All of the unit membranes measure between 70-100 Å in thickness as has been found by several authors (Afzelius, 1955; Wischnitzer, 1958, 1960; Barnes and Davis, 1959). Some of the nuclear membranes have remained in the in-vivo bi-membrane state and seem to have their perinuclear cisternae intact. The width of the perinuclear cisterna seems to vary from 150 Å to 450 Å. These dimensions agree with those given by Watson (1955, 1959), Wischnitzer (1960), Barnes and Davis (1959), and Baud (1959).

The fact that the homogenized nuclear preparation still contained many unbroken nuclei suggests that the nuclear envelope fragments were not subjected to intense shearing forces. Their frequent separation, evident in the electron micrographs, is evidence that the two unit membranes of the nuclear envelope are not tightly bound. This suggests that the content of the perinuclear cisterna is a free-water space since it shows very weak adhesive properties. Another finding which supports this hypothesis is that the electron micrographs (Figures 13-19) show ferritin adhering to both sides of all of the individual membranes, even those which seem to have their cisternae intact. Since these negatively-charged particles are adherent to the membranes, it is suggested that the nuclear membranes are positively-charged, as Risueño *et al.* (1965) and Feldherr (1964) suggested. Figure 18 shows the

ferritin equilibrated within the perinuclear cisterna. A free-water space would allow such a free diffusion.

Two micrographs reveal nuclear envelopes which appear to contain nuclear pore areas. The areas of discontinuity within the nuclear envelopes measure 1200 Å and 880 Å (in Figures 18 and 19, respectively) wide. These dimensions agree with previous measurements of the nuclear pore areas (Watson, 1959; Gall, 1959; Barnes and Davis, 1959; Wischnitzer, 1958). Annuli were not revealed at these pore areas, as might have been expected with $KMnO_4$ fixation (Gall, 1959; Merriam, 1961). The diaphragm is not clearly distinguishable in either of the nuclear pore areas in Figures 18 and 19. This was expected according to Merriam (1961) who found that $KMnO_4$ fixation removed the diaphragm.

The conclusion that the perinuclear cisterna is a free water space, available for diffusion of particles such as ferritin, supports the work of Palay (1960) wherein he found the contents of the perinuclear cisternae fluid enough to allow the unobstructed passage of fat droplets. It also supports the work of Langendorf et al. (1964) who found that the transfer of Na^+ from the extracellular space to the nucleus occurred through the endoplasmic reticulum which directly communicated with the pericellular and intranuclear spaces. Palade (1955, 1956), Terzakis (1965), Watson (1955), and Baud (1959) also contend that the contents of the perinuclear cisternae communicate with the contents of the endoplasmic reticulum

cavities. The reticular cavities are believed to be water spaces since they have been shown to communicate freely with the cytoplasmic matrix, believed to be a free water space (Palade, 1955, 1956; McAlear and Edwards, 1959; Langendorf et al., 1964). The intercommunication of these three systems of spaces, the endoplasmic reticulum cavities (a free water space), the cytoplasmic matrix (a free water space), and the perinuclear cisternae, suggests that the perinuclear cisterna is a free water space.

The perinuclear cisterna has, however, been interpreted in many micrographs as appearing different (usually lighter) than the cytoplasmic matrix (Watson, 1955) which would suggest that the composition of the two spaces is not the same.

The evidence given by Green (1959), Green and Fleischer (1962), and Parsons (1963), who determined that the "intracristal" space of mitochondria is not a free water space but is composed of solid material of lipid and structural protein, also suggests that the perinuclear cisterna, another space between a double membrane structure, might not be a freely-diffusible water space. The findings in many permeability studies have indicated that the penetrant does not enter the perinuclear cisternae, e.g., Feldherr (1964, 1965). This suggests that the perinuclear cisterna is not a freely diffusible water space. The findings of this study, however, suggest that the perinuclear cisterna of isolated nuclear envelopes is a free water space, allowing the entrance and equilibration

of ferritin particles and serving as a separation point for the paired nuclear membranes.

SUMMARY

A method for isolation of plasma membranes has been successfully adapted to the isolation of nuclear envelopes, producing a good preparation and satisfactory yield. Investigation of the nuclear envelopes by electron microscopy has indicated that the perinuclear cisterna is a free water space, available for diffusion of water-suspended particles, and that the two nuclear membranes are held together by very weak bonds.

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